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Role of the *PAS1* Gene of *Pichia pastoris* in Peroxisome Biogenesis

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Abstract. Several groups have reported the cloning and sequencing of genes involved in the biogenesis of yeast peroxisomes. Yeast strains bearing mutations in these genes are unable to grow on carbon sources whose metabolism requires peroxisomes, and these strains lack morphologically normal peroxisomes. We report the cloning of *Pichia pastoris PAS1*, the homologue (based on a high level of protein sequence similarity) of the *Saccharomyces cerevisiae PAS1*. We also describe the creation and characterization of *P. pastoris pas1* strains. Electron microscopy on the *P. pastoris pas1* cells revealed that they lack morphologically normal peroxisomes, and instead contain membrane-bound structures that appear to be small, mutant peroxisomes, or "peroxisome ghosts." These "ghosts" proliferated in response to induction on peroxisome-

requiring carbon sources (oleic acid and methanol), and they were distributed to daughter cells. Biochemical analysis of cell lysates revealed that peroxisomal proteins are induced normally in *pas1* cells. Peroxisome ghosts from *pas1* cells were purified on sucrose gradients, and biochemical analysis showed that these ghosts, while lacking several peroxisomal proteins, did import varying amounts of several other peroxisomal proteins. The existence of detectable peroxisome ghosts in *P. pastoris pas1* cells, and their ability to import some proteins, stands in contrast with the results reported by Erdmann et al. (1991) for the *S. cerevisiae pas1* mutant, in which they were unable to detect peroxisome-like structures. We discuss the role of *PAS1* in peroxisome biogenesis in light of the new information regarding peroxisome ghosts in *pas1* cells.

PEROXISOMES are single membrane-bound organelles ~0.2–1.0 microns in diameter. They are found in virtually all eukaryotic cells and, depending on cell type and growth conditions, vary in abundance from two to several hundred per cell (Lazarow and Fujiki, 1985). The importance of peroxisomes in humans is demonstrated by the class of peroxisomal disorders of which Zellweger syndrome is an example. Affected individuals usually die within several years of birth, and cells from affected individuals have been shown to have non-functional peroxisomes (for review see Lazarow and Moser, 1989).

While the biochemistry of peroxisomes has been well-characterized (Van den Bosch et al., 1992), less is known about peroxisome biogenesis (Subramani, 1993). Peroxisomes are thought to arise from preexisting peroxisomes, just as mitochondria are believed to be derived from preexisting mitochondria (Borst, 1989). This is supported by electron micrographs showing budding peroxisomes (Osumi et al., 1975; Veenhuis et al., 1978). Additionally, peroxisomes contain no protein synthesis machinery, and all peroxisomal matrix and membrane proteins are synthesized on free polyosomes and then imported into the organelle (Subramani,

1993). Given these constraints, cells must do several things to produce and propagate functional peroxisomes: (a) peroxisomal proteins must be synthesized and imported into the organelle; (b) the correct substrates and cofactors must be imported into the organelle; (c) membrane material consisting of lipids and proteins must be transported to peroxisomes so that they can grow and divide; and (d) peroxisomes must be segregated to daughter cells during cell division.

To identify and characterize the proteins involved in these processes, mutants with generalized peroxisomal defects have been generated in several organisms. This approach has led to the cloning and sequencing of several genes essential for peroxisome biogenesis (see Subramani, 1993). Of these genes, only *P. pastoris PAS8* (McCollum et al., 1993) and the homologous *S. cerevisiae PAS10* (van der Leij et al., 1993) have been assigned a function that is clearly related to peroxisome biogenesis. In *pas8 P. pastoris* cells and in *pas10 S. cerevisiae* cells, proteins normally targeted to the peroxisome by a COOH-terminal PTS1 (peroxisome-targeting signal 1): a COOH-terminal -SKL tri-peptide or variant that has been shown to direct proteins to peroxisomes in

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1. **Abbreviations used in this paper:** DHAS, dihydroxyacetone synthase; PTS, peroxisome-targeting signal; SDH, succinate dehydrogenase; SC, synthetic medium/citrate; SD, /dextrose; SE, /ethanol; SG, /glycerol; SL, /lactate; SM, /methanol; SOLT, /oleic acid/Tween 40; YP, yeast extract with peptone; YPD, /dextrose; YPM, /methanol; YPOLT, /oleic acid/Tween 40.

several different organisms [Gould et al., 1989, 1990a, b; Aitchison et al., 1991]) are not imported into the peroxisome. Additionally, the PAS8 protein has been shown to bind the -SKL tripeptide (McCollum et al., 1993).

We have chosen to study peroxisome biogenesis in the methylotrophic yeast *Pichia pastoris*. It has two features that make it useful for this: (a) it grows well on oleic acid or methanol, and growth on either of these carbon sources requires, and causes a marked proliferation of, functional peroxisomes and peroxisomal enzymes; and (b) peroxisomes in oleate- or methanol-induced wild-type *P. pastoris* cells are easily identified as peroxisomes by immunofluorescence or electron microscopy (Gould et al., 1992).

Here we report the cloning of the *P. pastoris* homologue of the *S. cerevisiae* *PAS1* and the creation and characterization of *P. pastoris* *pas1* strains. Our results build on those reported for the *S. cerevisiae* *PAS1* gene (Erdmann et al., 1991). Unlike the *pas1* mutant of *S. cerevisiae* in which no morphological or biochemical evidence was found for the existence of peroxisome "ghosts," our data show the presence of these ghosts in the *pas1* mutant of *P. pastoris*. We also demonstrate that the peroxisome ghosts of the *pas1* mutant of *P. pastoris* are capable of importing some matrix proteins but not several others. Our data shed new light on the phenotype of the yeast *pas1* mutant and suggest that the *PAS1* protein may play a direct or indirect role in delivering membrane material to developing peroxisomes.

Materials and Methods

Bacterial Strains

The *Escherichia coli* strain DH5 α F' was the most frequently used strain. Miniprep DNA samples were prepared using a modified alkaline lysis procedure (Zhou et al., 1990). Other recombinant DNA techniques were performed essentially as described previously (Sambrook et al., 1989).

Yeast Strains

The *P. pastoris* wild-type strain (21-1), the arginine-requiring (GS190) and histidine-requiring (GS115) strains (accession numbers Y-11430, Y-18014, and Y-15851, respectively) were obtained from the Northern Regional Research Laboratories (Peoria, IL). We refer to these strains as PPY1, PPY3, and PPY4, respectively. PPY12 (*arg4 his4*) was generated previously by our group (Gould et al., 1992). The PPY300 (*arg4 his4 pas1::ScARG4*) and the PPY301 (*arg4 his4 Δ pas1::ARG4*) strains were developed for this study as described below. *P. pastoris* PPY20 (*arg4 pas1-2*) was isolated by Dan McCollum (Gould et al., 1992). PPY115 (*arg4 his4 Δ pas8::ARG4*) (McCollum et al., 1993) is referred to as *Δ pas8* in this paper.

Yeast Culture

Standard rich medium for growth of *P. pastoris* was YPD medium (1% yeast extract, 2% bacto-peptone, 2% dextrose). Defined synthetic medium consisted of yeast nitrogen base at 0.67% wt/vol supplemented with carbon sources to a final concentration of either 2% dextrose (SD), 0.5% methanol (SM), 1% citrate (SC), 1% lactate (SL), 1% glycerol (SG), or 0.2% oleic acid/0.02% Tween 40 (SOLT). For auxotrophic strains requiring arginine or histidine or both, the required amino acids were included at 40 μ g/ml.

Culturing, mating, sporulation, and random spore analysis were done as previously described (Gould et al., 1992).

Electron Microscopy

Electron microscopy was performed as described previously (Gould et al., 1992). However, after preculture of cells in defined dextrose medium (SD), cells were induced for 4 h in either SM or SOLT media, except for the cells shown in Fig. 6a, which were cultured for 7 h in YPM (1% yeast, 2% bac-

topeptone, 0.5% methanol) after preculture in YPD. These conditions are sufficient for induction of peroxisomes and peroxisomal proteins.

Preparation of Crude Yeast Cell Lysates

Cells were grown exponentially in SD and then switched to SM or SOLT for the desired length of peroxisome induction. Crude cell lysates were prepared by glass-bead lysis. This was performed essentially as previously described (Sambrook et al., 1989) except that the lysis buffer was 62.5 mM Tris-Cl (pH 8.7), 5 mM EDTA, 2 mM PMSF, and lysates were not cleared of debris before use in experiments.

Sucrose Gradient Purification of *P. pastoris* Organelles

Fractionation of *P. pastoris* cells into an organellar pellet and supernatant was performed as described previously (Gould et al., 1992), but with the following changes. Cells were grown in a semi-rich inducing media (1% bacto-peptone wt/vol, 2% wt/vol yeast extract) containing oleic acid and Tween 40 (0.2% vol/vol of a 9:1 mix of oleic acid and Tween 40, respectively, YPOLT) before being fractionated. Peroxisomes were purified from this organelle pellet on a sucrose gradient as previously described (Nuttley et al., 1990).

Sucrose gradients were drained with the aid of a peristaltic pump and fractions were assayed for catalase and succinate dehydrogenase (SDH) activities as described previously (Sottocasa et al., 1967; Leighton et al., 1968).

In Figs. 9 and 10, the following steps were taken so that the relative band intensities seen for a particular protein in Fig. 9 vs. Fig. 10 would reflect the relative amounts of that protein in the wild-type (Fig. 9) and the *Δ pas1* (Fig. 10) peroxisomes: (a) 8 mg of crude organellar protein was loaded onto each of the 40 ml gradients (wild type and *Δ pas1*); (b) each gradient was drained into 40 fractions; (c) an equal volume (35 μ l) of every fourth fraction (1, 4, 7...40) of each gradient was loaded onto 8% SDS-PAGE gels (one gel per gradient); (d) these gels were run and transferred to nitrocellulose equivalently; (e) the Western blot filters for each gradient were incubated at the same time and in the same vessels during Western blotting; and (f) for blots with a given antibody, films were developed by ECL for the same amount of time. Film negatives were made from these films with the camera at a fixed setting, and these negatives were printed at the same exposure setting.

Western blots were done using antisera to thiolase (gift of Wolf Kunau, Ruhr-Universität Bochum, Germany), *P. pastoris* acyl-CoA oxidase (A. P. Spong and S. Subramani, manuscript in preparation), the *P. pastoris* PAS8p (McCollum et al., 1993) and dihydroxyacetone synthase (DHAS). Antisera to the carboxy-terminal PTS1 peptides, SKL (Gould et al., 1990b) and AKI (gift of R. Rachubinski, University of Alberta, Canada), and antisera to the *S. cerevisiae* multifunctional enzyme (gift of Wolf Kunau), which recognizes the analogous protein, trifunctional enzyme, of *P. pastoris*, were also used. In 8% SDS-PAGE, *P. pastoris* proteins run at the following molecular masses: thiolase (47 kd), acyl-CoA oxidase (72 kd), trifunctional enzyme (97 kd), PAS8 (68 kd), DHAS (76 kd). The anti-multifunctional enzyme antibody is referred to as anti-trifunctional enzyme antibody. Blots shown in Figs. 9, 10, 11, and 12 were developed using the ECL (Amersham Corp., Arlington Heights, Illinois) system, but all others were done according to Sambrook et al. (1989).

Two-step Sucrose Flotation Gradients

Fractionation of *P. pastoris* cells into an organellar pellet and supernatant was performed as described above. This pellet was resuspended in 400 μ l sucrose gradient buffer, loaded onto a 5-ml sucrose gradient, spun for 2.5 h at 92,000 g, and fractionated into 12 fractions of equal volume. 200 μ l of a fraction of density within the range at which purified wild-type peroxisomes are expected to be found (in these gradients, \sim 1.18–1.23 g/cm³) was transferred to the bottom of a Beckman ultra-clear centrifuge tube, overlaid with 2.4 ml 60% sucrose, overlaid again with 2.4 ml 35% sucrose, and the sample was spun at 170,000 g for 18 h and harvested from the bottom (fraction 1) to top (fraction 12 [wild type], fraction 13 [*Δ pas1*]) in fractions of \sim 0.5 ml (Paravicini et al., 1992). Flotation gradient fractions were assayed for sucrose density and for catalase and SDH activities across the gradient. These fractions were also immunoblotted with the anti-acyl-CoA oxidase, anti-trifunctional enzyme, anti-PAS8, and anti-thiolase antibodies described above. After each flotation gradient had been loaded with a portion of the fraction from each 5-ml purification gradient, each purification gradient was assayed across the gradient for sucrose density and for catalase and SDH activities. The fractions loaded on the flotation gradients had no mito-

chondrial contamination (no SDH activity) and had sucrose densities of 1.21 and 1.20 g/cm³ for the wild-type and $\Delta pas1$ fractions, respectively. These fractions were also immuno-blotted with the anti-acyl-CoA oxidase, anti-trifunctional enzyme, anti-PAS8, and anti-thiolase antibodies to confirm the quality of the purification gradients.

Isolation and Sequencing of the *PAS1* Gene

A polymerase chain reaction (PCR) was performed on a *P. pastoris* genomic DNA library contained in the plasmid pFL20 (Losson and Lacroute, 1983; Gould et al., 1992). Primers "A" and "B," with Universal Wobble Code sequences 5'-GGG GGA TCC NGG NTG YGG NAA RAC NYT NYT NGC-3' and 5'-CCC GAG CTC ARN CKN CCN GGN CKN ARN ARN GC-3', respectively, were obtained from Operon Technologies, Inc. (Alameda, CA). The PCR product from this reaction was ligated into pUC19, and the resultant plasmids were transformed into DH5 α *E. coli* cells. DNA was isolated from the transformants and sequenced, allowing identification of a clone, pJAH11, that contained a fragment of the *P. pastoris PAS1*. This fragment was subsequently used as a probe to screen by hybridization (performed according to Sambrook et al., 1989) the *P. pastoris* genomic library and isolate a clone (pJAH12) which contained the full-length *P. pastoris PAS1* gene.

Sequencing was performed by subcloning fragments into pUC19 (New England Biolabs, Inc., Beverly, MA) and pBluescript/KS II (Stratagene, La Jolla, CA). Nested deletions were also generated using Exonuclease III and SI nuclease as described previously (Sambrook et al., 1989). The Sequenase II system (United States Biochemical, Cleveland, OH) which is based on the dideoxynucleotide chain-termination method was used for nucleotide sequencing. Primers T3 or T7 (for pBluescript-based plasmids) and New England Biolabs primer no. 1212, 1201, 1220, and 1211 (for pUC19-based plasmids) were used together with denatured, double-stranded DNA templates. Both strands were entirely sequenced.

Construction of *pas1* Strains

The *pas1* disruption (PPY300 [*arg4 his4 pas1::ScARG4*]) strain was constructed by homologous recombination of the *S. cerevisiae ARG4* gene into the *P. pastoris PAS1* locus of strain PPY12. A targeting construct was made in the pUC19 vector by first ligating the 2.4-kb BglII fragment of *PAS1* coding sequence into the BamHI site of pUC19 to create pJAH13. Subsequently, the *S. cerevisiae ARG4* gene was removed from pSG464 with SalI and blunt-end ligated into the XhoI site of pJAH13 to create pJAH14. The targeting DNA was released from the vector by digestion with the restriction enzymes SmaI and XbaI. The released fragment was isolated from an agarose electrophoresis gel according to Sambrook et al. (1989) and introduced into PPY12 (*arg4 his4*) by electroporation (Bio-Rad Laboratories, Hercules, California). (The 2.4-kb fragment and the XhoI site of *PAS1* are shown in Fig. 7).

The *pas1* deletion (PPY301 [*arg4 his4 $\Delta pas1::ARG4$*]) was constructed in a similar fashion to that described for PPY300 except that the targeting construct used is shown in Fig. 7. The plasmid containing the targeting construct (pJAH21) was pBluescript/KSII-based, and the targeting DNA was released from this plasmid by the enzymes KpnI and BamHI. Correct targeting of these fragments was confirmed by Southern analysis (Sambrook et al., 1989).

Strain PPY300 was characterized as a peroxisome assembly (*pas*) mutant because it did not grow on SM or SOLT, but it did grow on SC, SL, SE, and SG (since yeast require functional mitochondria to utilize glycerol, PPY300 must have functional mitochondria). PPY300 and PPY301 were equally defective for all peroxisomal functions tested.

Complementation of the *pas1* Mutants

A 5.2-kb SmaI to BamHI fragment of pJAH12 was cloned into the *P. pastoris* autonomously replicating plasmid pSG464 (Gould et al., 1992). This fragment contained the entire *PAS1* open-reading-frame together with ~900 bp 5' of the initiator ATG and ~500 bp 3' of the stop codon. (This fragment also contained at its 5' end 400 bp of the pFL20 vector which could not be cut away from the insert.) The resulting plasmid, pJAH15, was introduced into strain PPY20 (*arg4 pas1-2*) by electroporation. Transformants were selected by their ability to grow on plates without arginine. All transformants tested were able to grow on SOLT and SM plates.

In order to complement the PPY300 and PPY301 strains (both histidine auxotrophs), a complementation construct containing the *P. pastoris HIS4* gene was constructed. This was accomplished by first blunt-end ligating the *P. pastoris PARS2* sequence (Cregg et al., 1985; Gould et al., 1992) into the ClaI site of pYM5, a plasmid consisting of the *P. pastoris HIS4* gene cloned into the BamHI site of pBR322. This new construct, pJAH16, was digested with EcoRV and blunt-end ligated with the 5.2-kb SmaI to BamHI fragment of pJAH12 described above. The resulting plasmid, pJAH17, was introduced into strains PPY300 and PPY301 by electroporation. Transformants were selected by their ability to grow on plates without histidine. All transformants tested were able to grow on SOLT and SM plates.

Computer Analysis of Sequences

Sequences were analyzed using the Macvector software (IBI, New Haven, CT) and also FASTA (Pearson and Lipman, 1988).

Results

The Yeast *P. pastoris* Contains a Homologue of the *S. cerevisiae PAS1* Gene

Fig. 1 shows the region of high homology between the *S. cerevisiae PAS1* (Erdmann et al., 1991) and *S. cerevisiae SEC18* proteins (Eakle et al., 1988). We reasoned that a protein domain shared between these two proteins would also be found in the *P. pastoris PAS1* homologue. Accordingly, we designed degenerate PCR primers corresponding to the DNA encoding this domain. Repeatedly, the A and B primers amplified fragments of ~380 and 350 bp from a *P. pastoris* genomic library. These were the only abundant reaction products seen (data not shown).

As can be inferred from the protein alignment in Fig. 1, a PCR product from the *P. pastoris PAS1*-homologue DNA



Figure 1. Highly homologous regions of *S. cerevisiae* (Sc) SEC18 and PAS1. Oligonucleotides fully degenerate for the DNA encoding the peptides indicated by arrows A and B were used to PCR-amplify *P. pastoris* genomic DNA. Identities are indicated by vertical lines and similarities are indicated with dots. Two dots represent highly conserved residues, a single dot represents weakly conserved residues.

The *PASI*-derived fragment was then used in a hybridization-screen of a *P. pastoris* genomic library contained in the pFL20 vector (Losson and Lacroute, 1983; Gould et al., 1992). A clone containing a 7.1-kb genomic insert was obtained, and sequencing of a portion revealed the clone to contain the entire *P. pastoris PASI* gene (Fig. 2).

[illegible]

Figure 2. Nucleotide sequence and deduced amino acid sequence of the *P. pastoris* (*P.p.*) *PAS1* gene. *PAS1* nucleotide and deduced amino acid sequence. The translation of the *PAS1* sequence from the ATG at nucleotide 1 (underlined) to the termination codon is shown in one letter code below the first nucleotide of each codon in the sequence. The two putative ATP-binding domains are double-underlined. The *PAS1* open reading frame is 3471 base pairs long, encoding a 1157-amino acid protein with predicted molecular mass of 127 kD.

A comparison of the sequences of the *P. pastoris* PAS1 and the *S. cerevisiae* PAS1 proteins (Fig. 3) shows that the proteins share 39% sequence identity and 60% similarity. The *P. pastoris* protein is 114 amino acids longer than the *S. cerevisiae* protein, and regions of strong homology are seen in the line-up at *P. pastoris* residues 458–705 and 771–1052. These regions contain consensus ATP-binding sites at *P. pastoris* residues 523–530 and 840–847, and these are the regions which place PAS1 in the growing family of ATP-binding proteins (Kunau et al., 1993). The family now includes PAS1, CDC48 (Frohlich et al., 1991), and SEC18 from *S. cerevisiae*; PAS5 (Spong and Subramani, 1993) and its homologues, PAY4 (Nuttley et al., 1993) and PAS8 (Voorn-Brouwer et al., 1993), from the yeasts *P. pastoris*, *Yarrowia lipolytica*, and *S. cerevisiae*, respectively; NSF (the SEC18 homologue [Wilson et al., 1989]), VCP (the CDC48 homologue [Koller and Brownstein, 1987]), and p97 (Peters et al., 1990) from vertebrates; and the virally encoded TBP (Nelbock et al., 1990).

P. pastoris PAS1 and *P. pastoris* PAS5 (Fig. 4) share 49% similarity and 26% identity. Not surprisingly, the *P. pastoris* *pasl* and *pas5* mutants have similar phenotypes (but they do belong to different complementation groups). These similarities are addressed in the Discussion.

The *P. pastoris* PAS1 Is Required for Functional Peroxisomes

The strain PPY300 (*arg4 his4 pasl::ScARG4*), containing a disruption of the *PAS1* gene, differed phenotypically from PPY12 (*arg4 his4*) in several ways related to peroxisome function. (Hereon, PPY300 will be referred to as *pasl::ARG4*. See Materials and Methods for details of strain construction.)

Wild-type *P. pastoris* grows on either oleic acid or methanol as sole carbon source, and growth on either substrate causes substantial peroxisomal proliferation (Gould et al., 1992). Electron micrographs of wild-type, oleic acid-grown cells contain numerous mitochondria-sized peroxisomes, while wild-type cells grown on methanol contain several clustered, huge peroxisomes (Fig. 5).

The *pasl::ARG4* strain, however, was unable to grow on either oleic acid or methanol as sole carbon source, and electron micrographs of *pasl::ARG4* cells cultured on either carbon source showed no structures comparable to the peroxisomes seen in oleic acid- or methanol-induced wild-type cells (Fig. 6, *a* and *b*). Notably, the induced *pasl::ARG4* cells did contain some structures that resembled the peroxisomes seen in methanol-induced wild-type cells at early time points

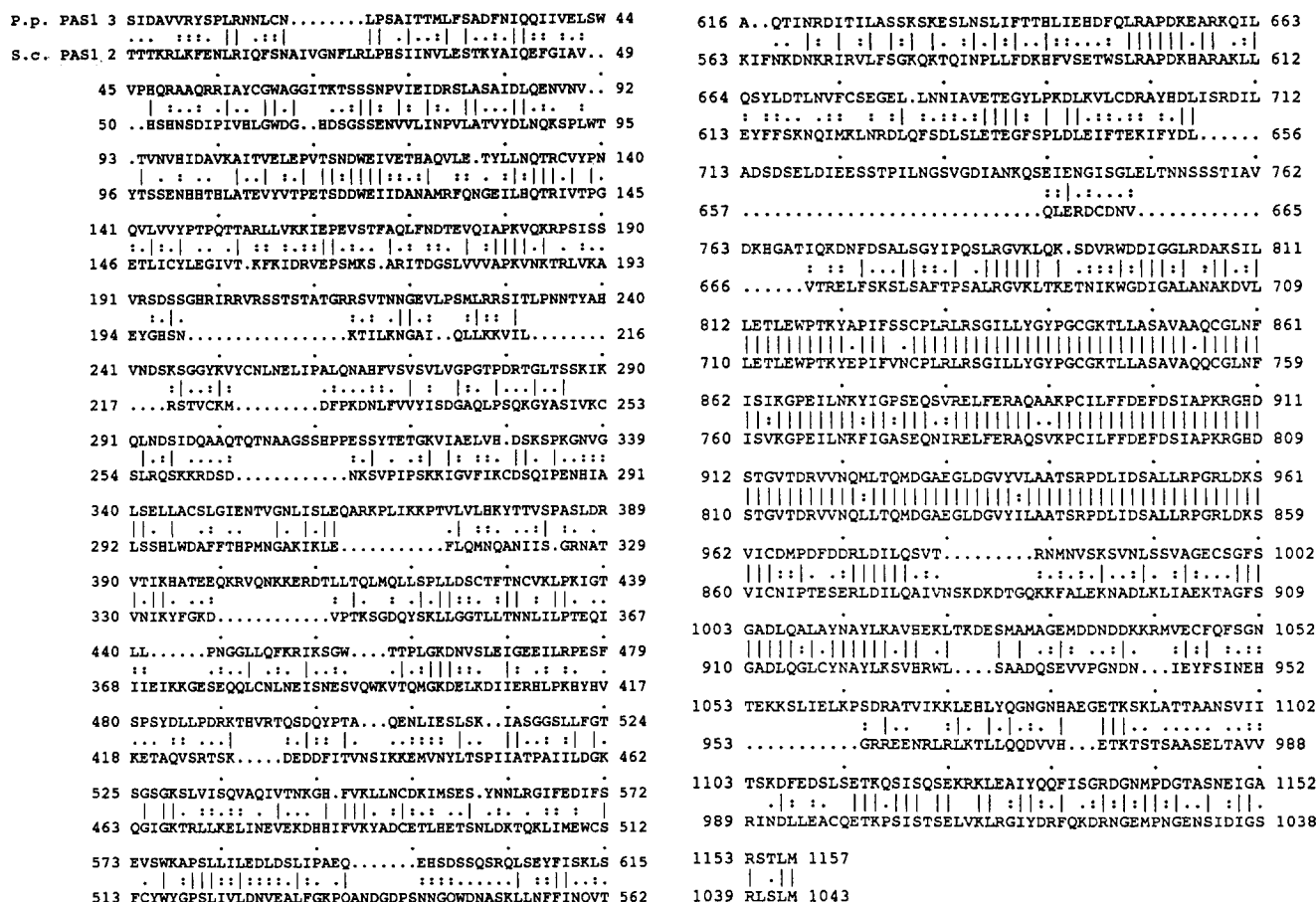


Figure 3. Comparison between *P. pastoris* (*P.p.*) PAS1 and *S. cerevisiae* (*S.c.*) PAS1 proteins. Sequences were aligned using the BEST-FIT program.

p.p. PAS1 20 LPSAITTMLFSADFNQIIIVELSWPHQRAAQRIAYCGWAGGITKTS 69
 p.p. PAS5 1 MPGITESTVGTGPVLAHVITVDFPDYASERAFSLDTELLFEDYANGSK 50
 70 SNPVIEIDRSASAIQDQENVVTVNVHIDAVKAITVELEPVT....SN 114
 51 SGLTLISIQLGSSSL.FNEFQTFFVYSESEQLPNTVNLNMGNIIDYS 99
 115 DWEIVETHAQVLETYLLNQTRCVYFNQVLVYPTQTARLLVKKIEPV 164
 100 DFTVDSGYVARVDSLVKLDL.VIISVPEVYSLASQSQHQLVDILGND 147
 165 STFAQLFNDTEVQIAPKVKQKPSISSVSDSSGHRIRRVRSSTSTA...T 211
 148 QHTVIRQGDYNDKNGKISLCEPTDQGFLESTTKIIVKENSINLPLLDQ 197
 212 GRRSVTNNGEVLPSMLRRSIT..LPNNYAHVNDKSKSGG..YKVCYNLNE 257
 198 SQDGSINYEENVKNNLEHSISNTYFSLNLDPENQITTTGVEFSVKC.LDS 246
 258 LIPALQNAHFVS.....SVLVGPGTDPDRTGL.....TSSKIK 290
 247 PISVRKTAISVAVHSEDESSPKLVEEDISNEDTLLYAFCKTTELAKIG 296
 291 QLNDSIDQAAQTQNAAGSSHPPESSYTETGKV.IAELVHDSKSPKGNVG 339
 297 CLSGDGVKMSGQCCQTTTCNCESCPVOYRIRIHAFTDPNTYKGCYI 346
 340 LSELLACSGIENTVGNLISLEQAKPL....IKKPTVLVHKYTTV... 382
 347 LNPILSFNLN.NPKIVKLCPISIDPKRFELQGFHFSKFIPLAKQVTIARV 395
 383 .SPASLDR.....VTIKHATEEQKRVQNKERDITLLTQMLQ.LSPLL 423
 396 SSPVTLDRLTQLFLNLKTYFESGRKVLSD.....QLIPVPDITLL 438
 424 DSCITFNCVKL....PKIGTLLPNC.....GLLQFKRIKSGWTPLGKDN 464
 439 AKSIFSTYEKLGVDSSQFPTVIPEGKPDIAIAPKVTVEVSGELASASQF 488
 465 VSLEIGEELIRPESFPS.....YDLLPDRKTHVRQSD 498
 489 IIDPLTKMKMGSGVSCSPPKNSQHCNANYLQCGQMFSPNVSGVTST 538
 499 .QYPTAQENLIESL...SKIASGSLLPFTSGS...GKSLVISQVAQIVT 541
 539 FEYAKTLRLKIKATIDPSRLVNLQTTVLSSLSRAIGKSLVLVHSLA..LE 586
 542 NKGHFVKLLNCDKIMSESYNNLRGIFEDIFSEVSKWAPSLIL.EDLDSL 590
 587 CGVHLVEIDGVEVLNPSSEKSTIGTIRGKLDREVCGTPLIVFKHIEAL 636
 591 IPAEQEHSDSSQSRQLSEYFISKLQAOTINRDTILASSKSKESLNSLIF 640
 637 TKKSEQQKDSLAVKINEL....IDEYTAQPGVLFVASTNDSNLSEDLR 682
 641 TTHLIEHDFOLRAPDKAARKQILQSYLD....TLNVFCSEGL..... 679
 683 AK..FKFEIVLGVSEQERTLIFKYLIDFDQKTPKVTETGRELSPAPRN 730
 680 ..LNNAVETEGYLPKDLKVLCDRAYHDLISRDILADSDSELDEEST 726
 731 DLSLSSLSLQAGLTPRDLISIVENAKTLAVDRVESLAKHNTVSFEN... 777
 727 PILNGSVGDIANKQSEIENGISGLELTNNSSSTIAVDKKGATIQKDNFDS 776
 778 .MVYSGGYIKFPTPEDVEKSIINT.....ARNYFSDSIGAPRI... 813
 777 ALSGYIPQSLRGVQLQKSDVAVDDIGGLRDAKSILLETLEWPTYAPIFS 826
 814PNVKWEDVGLDVVDEILDITDMPMKHPFLS 846
 827 SCPLRLRSGILLYGYPGCGKTLASAVAAQCLNFIISIKGPEILNKYIGP 876
 847 N.GIKKRSGLIFYGPPGTGKTLAKAJATNFALNFFSVKGPPELLNMYIGE 895
 877 SEQSVRELFERAAQAKPCILFFDEFDSIAPKRGH...DSTGVTDVVNQML 924
 896 SEANVAKVFORARDAPKCVVDFEELDSVAFKRGKQSGSEGVMDRIVSQLL 945
 925 TQMD...GAELDGVVYLAATSRPDLIDSALLRPGLRDKSVICMPDFDD 971
 946 AELDQMSGGDGGCVFVVGATNRPDLLEALLRPGRFDKMLYLGVSDTHE 995
 972 RL.DILQSVTRNNVSKSVNLSSVAGECS.GFSGADLQALAYNAYLKAVH 1019
 996 KQSKIMEALSRRKPHLHPSVDLDKVAESCPFTTGTADFYALCSDAMLNA.. 1043
 1020 EKLTKDSEMAAGEMDNDKGRMVECFQFSQNTKESLSLIELKPSDRAIV 1069
 1044 .MTR.....IANTVDEK..IKRYNEELPEKSVSTRWVFD..... 1075
 1070 IKKLEHLYQNGNHAEGETASKLATTANSVITTSKDFEDSLSETKQISIS 1119
 1076NVATKEDIDLVLTLEDFDKSRKELVPSVS 1104
 1120 QSEKRLKLEAIYQFISGRD.....GNMPDGTASNEIGARSTLM 1157
 1105 AEELDHYLRVRQNFEGGKQVQENGQTEHFSNGSANNHITFGDEQV 1152

Figure 4. Comparison between *P. pastoris* PAS1 and PAS5 proteins. Sequences were aligned using the BEST-FIT program.

of induction (data not shown). (These putative "peroxisome ghosts" will be discussed later.)

Thus, the *pas1::ARG4* disruption confirmed that the PAS1 protein is required for functional peroxisomes in *P. pastoris*, and it defined *P. pastoris pas* (peroxisome assembly) mutants as being: (a) unable to grow on oleic acid or methanol as sole carbon source; and (b) having no morphologically normal peroxisomes.

Later, to be sure that the disruption removed all PAS1 activity, we constructed a strain, PPY301 (*arg4 his4 Δpas1::ARG4*), with a near-complete deletion of the PAS1 gene (Materials and Methods and Fig. 7). The *pas1::ARG4* and the *Δpas1* strains were equally deficient for all aspects of peroxisomal function tested. (Hereon, PPY301 will be referred to as *Δpas1*.)

We were able to restore functional peroxisomes in both the *pas1::ARG4* strain (Fig. 6, c and d) and the *Δpas1* strain (data not shown) by transformation with extrachromosomal plasmids that contained the full *P. pastoris PAS1* open reading frame and ~900 base pairs of DNA 5' of the ATG initiation codon.

Peroxisomal Proteins Are Induced Normally in *pas1::ARG4* Cells

As mentioned earlier, the production of peroxisomal proteins required for growth on oleic acid or methanol is greatly increased when *P. pastoris* is grown on either of these carbon sources. It is thought that the increase in size of wild-type peroxisomes during induction is due in part to increased import of these proteins (Hazeu et al., 1975; McCollum et al., 1993). Thus, a defect in peroxisomal protein induction could cause the morphological and growth defects seen in the *P. pastoris pas1* strains.

We tested this possibility by comparing the levels of peroxisomal protein produced in whole-cell lysates of wild-type and *pas1::ARG4* cells.

The *pas1::ARG4* strain and the wild-type strain produced thiolase (a peroxisomal matrix protein [Kunau et al., 1988]), PAS8 (a peroxisome-membrane-associated protein [McCollum et al., 1993]), and DHAS (dihydroxyacetone synthase, a peroxisomal matrix protein) at similar levels (Fig. 8). Additionally, catalase and methanol oxidase (both peroxisomal matrix proteins) were induced normally in the *pas1::ARG4* strain (data not shown). Therefore, PAS1 is not involved in peroxisomal protein induction in *P. pastoris*.

Δpas1 Cells Contain Peroxisomal Ghosts

Erdmann et. al (1991) were unable to find peroxisome-like structures (ghosts) in their *S. cerevisiae pas1* strains by either electron microscopy or by biochemical examination of subcellular fractions. Interestingly, the *P. pastoris pas1* strains contain peroxisomal ghosts.

The electron micrographs in Fig. 6 show *pas1::ARG4* cells grown on methanol (Fig. 6 a) and oleic acid (Fig. 6 b). In these micrographs we have indicated structures that resemble mutant peroxisomes (McCollum et al., 1993; Spong and Subramani, 1993). These structures are commonly seen in *pas1* cells induced by methanol or oleic acid, but similar structures are only rarely seen in uninduced *pas1* cells or in

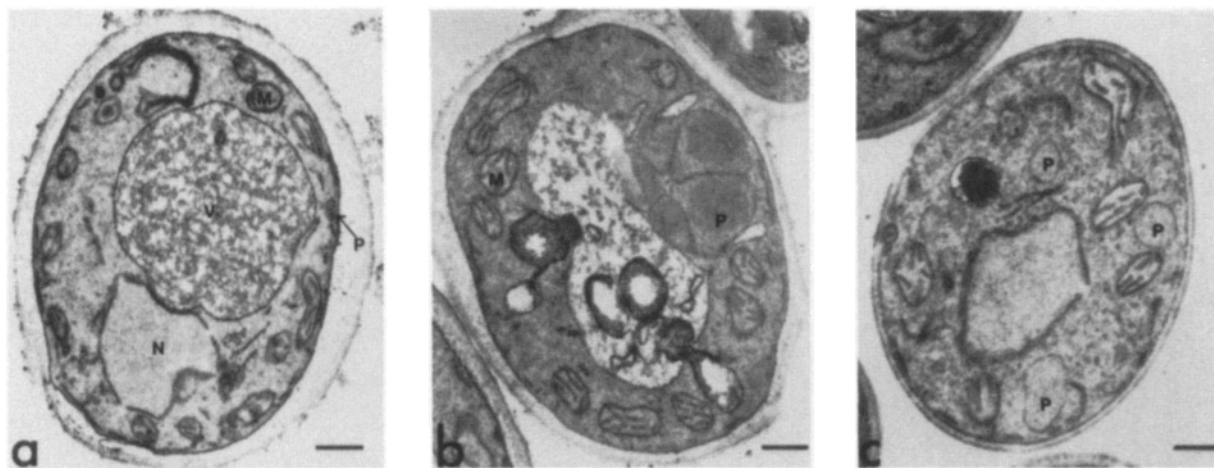


Figure 5. Growth of *P. pastoris* on methanol or oleic acid causes a massive proliferation of peroxisomes. (a) Wild-type *P. pastoris* grown in glucose medium. (b) Wild-type *P. pastoris* grown in methanol medium. Note the large, clustered peroxisomes (P). (c) Wild-type *P. pastoris* grown in oleic acid medium. Note the large, unclustered peroxisomes (P). M, Mitochondria; N, nucleus; P, peroxisome; V, vacuole. Bar, 500 nm.

uninduced wild-type cells. Interestingly, structures similar to the stacked organelles indicated as peroxisomes in Fig. 6 a are regularly seen in *P. pastoris* $\Delta pas5$ cells cultured in methanol media and in wild-type cells cultured in methanol media for short periods (less than 2 h) of time (Heyman and Monosov, unpublished observations).

To further examine the possibility that *P. pastoris pas1* cells contain mutant peroxisomes, we purified, then com-

pared, peroxisomes from oleic-acid induced wild-type and $\Delta pas1$ cells. The data presented below were obtained from analysis of two sucrose gradients: The "wild-type" and " $\Delta pas1$ " gradients were loaded with crude organellar pellets from wild-type and $\Delta pas1$ cells, respectively. Each 40-ml gradient was loaded with 8 mg total protein. During the characterization of the gradients for Figs. 9 and 10, care was taken in the Western blotting and in the preparation of nega-

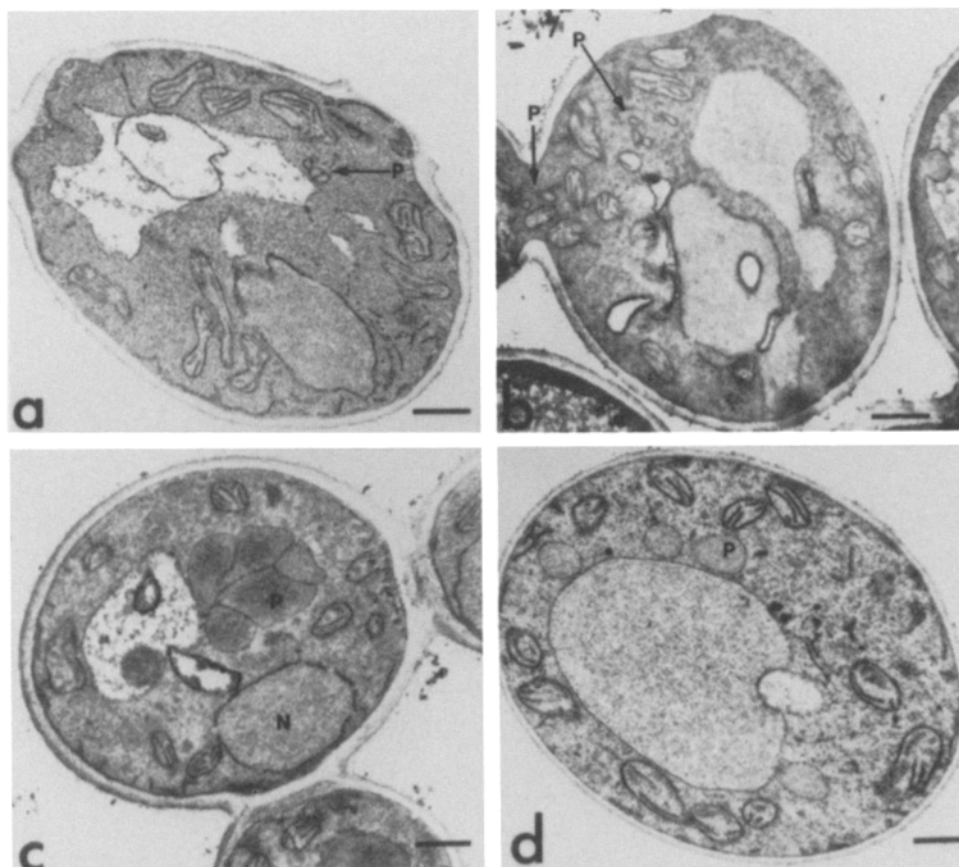


Figure 6. Methanol- or oleic-acid-induced *pas1::ARG4* cells contain structures that look like very small peroxisomes. Transformation with extrachromosomal copies of the *PAS1* gene restores normal peroxisomes to the *pas1::ARG4* strain. (a) *pas1::ARG4* cells cultured in methanol medium contain clustered, single-membrane-bound structures that resemble small peroxisomes (P). (b) *pas1::ARG4* cells cultured in oleic acid medium contain several small, single membrane-bound structures that are not seen in uninduced wild-type cells. *pas1::ARG4* cells harboring the *PAS1* gene on extrachromosomal plasmids contain morphologically wild-type peroxisomes (P) when cultured in (c) methanol, or (d) oleic acid. Bar, 500 nm.

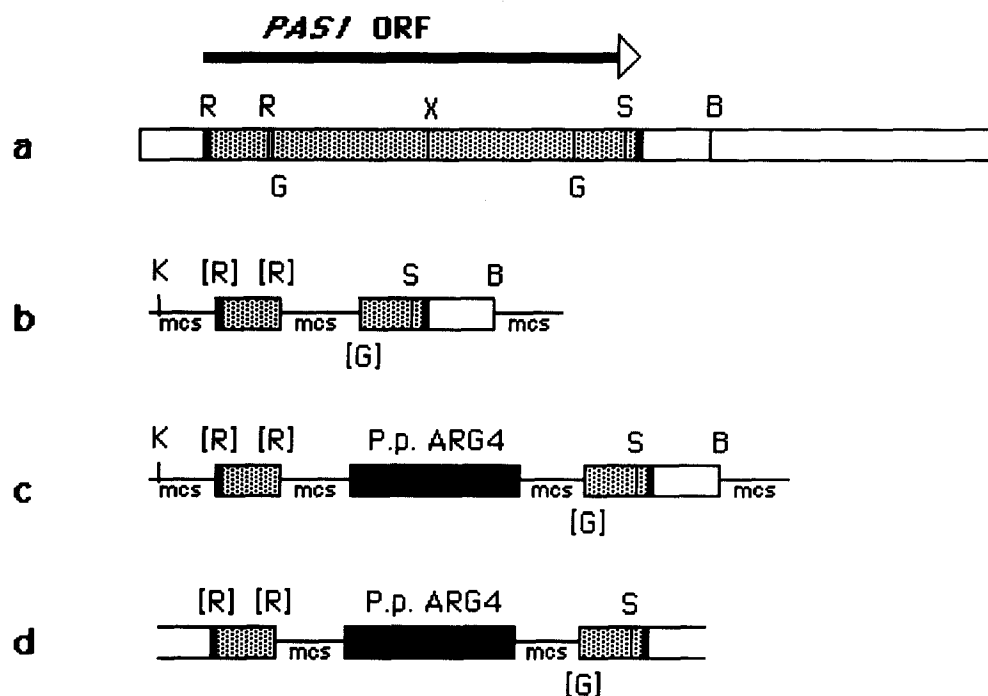


Figure 7. Deletion of the *PAS1* gene. The 5' 600-bp EcoRI (R) fragment (blunted) and the 3' 1200-bp BglII (G) to BamHI (B) fragment were cut from pJAH15 (a) and cloned consecutively into the XhoI (blunted and treated with phosphatase) and BamHI (treated with phosphatase) sites, respectively, of the pBluescript-IIS multiple cloning site (mcs), forming the construct in b. The *P. pastoris* *ARG4* gene was then excised from pAS100 (Spong and Subramani, 1993) with KpnI and EcoRV (a 2075-bp fragment), blunted, and ligated into the EcoRV (treated with phosphatase) site between the *PAS1* fragments (c). Thus, 2.4 kb of *PAS1* coding sequence was replaced with the *P. pastoris* *ARG4* gene. Digestion at the KpnI (K) and SmaI (S) sites was used to release the *PAS1*-

targeted *ARG4* gene, and this DNA was introduced into the genome by homologous recombination into PPY12, forming the $\Delta pas1$ locus depicted in d. Enzyme sites in brackets were destroyed during cloning. The *PAS1* coding sequence is shaded and bounded by heavy vertical lines.

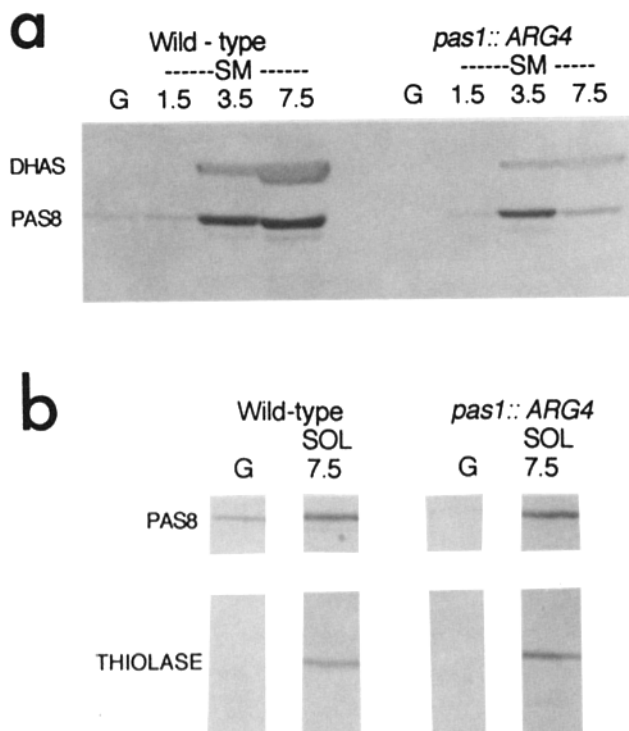


Figure 8. Growth on oleic acid or methanol causes normal induction of peroxisomal proteins in *pas1::ARG4* cells. Wild-type and *pas1::ARG4* cells were precultivated in yeast nitrogen base with 2% glucose (SG) and then shifted to either yeast nitrogen base with methanol (SM) or yeast nitrogen base with oleic acid and Tween (SOLT) for 1.5, 3.5, or 7.5 h. (a) The methanol-induced cells were immuno-blotted for the presence of DHAS and PAS8. (b) The oleic-

acid induced cells were immuno-blotted for the presence of PAS8 and thiolase.

Figure 9 summarizes experiments performed on sucrose fractions from a peroxisome purification gradient for wild-type cells. Catalase activity was seen primarily in fractions 13–25, with a peak in fraction 16, and SDH activity (mitochondrial marker) was seen in fractions 28–37, with peak activity in fraction 31 (Fig. 9 a). Western blots with antibodies against trifunctional enzyme (Fig. 9 b), acyl-CoA oxidase (Fig. 9 c), and thiolase (Fig. 9 d) show that these peroxisomal proteins also colocalized with catalase within the gradient. Blotting with anti-PAS8 antibody (Fig. 9 e) showed the PAS8 protein to have a bimodal distribution within the gradient: one peak coincided with the peroxisomal markers, and the other peak was likely to be the result of PAS8 protein running as a free protein or as a protein that sticks nonspecifically to mitochondria. (An experiment in which human serum albumin was added to an organelle pellet before sucrose gradient centrifugation showed that free protein runs at the top of these sucrose gradients; data not shown.)

Since four peroxisomal matrix proteins and one peroxisomal membrane-associated protein colocalized in fractions 13–25, and because there was virtually no SDH (Fig. 9 a) activity in these fractions, it is clear that intact peroxisomes, free of mitochondrial contamination, were purified in frac-

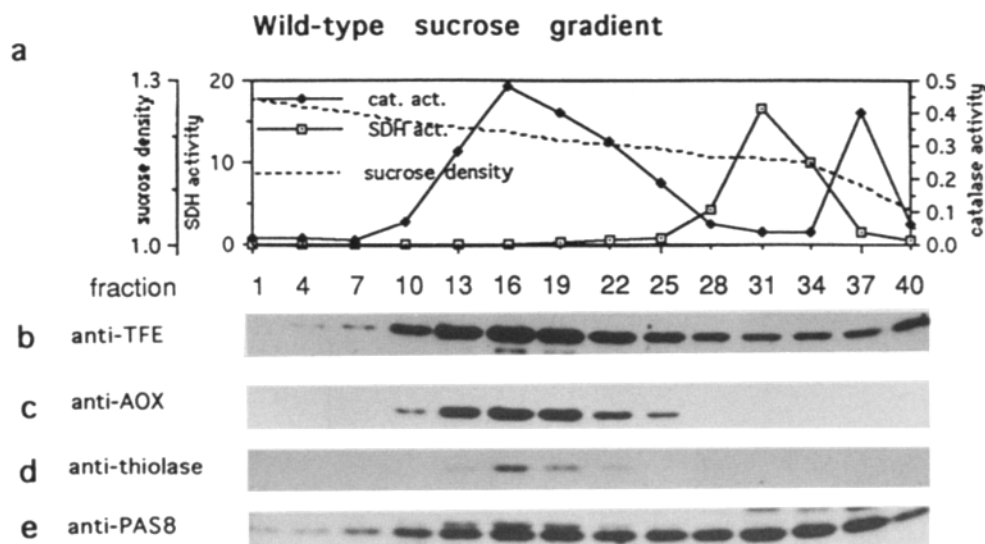


Figure 9. Purification of peroxisomes from wild-type cells on a sucrose gradient. Wild-type cells were induced in oleic acid for 18 h and the organelle pellet fraction (8 mg), consisting primarily of peroxisomes and mitochondria, was fractionated on a sucrose gradient as described by Nuttley et al. (1990). The gradient was drained into 40 1-ml fractions. (a) Gradient fractions assayed for catalase and SDH (succinate dehydrogenase) activity across the gradient. Catalase activity is expressed in Beaufay U/ml. SDH activity is expressed as the change in absorbance at 410 nm/min/ml of fraction. Density of fractions

tions in g/cm³. (b–e), results of Western blotting equal volumes (35 μ l) of every fourth gradient fraction (including the first and last fractions) with: (b) antisera to trifunctional enzyme (abbreviated “anti-TFE”); (c) antisera to acyl-CoA oxidase (abbreviated “anti-AOX”); (d) antisera to thiolase; and (e) affinity-purified antisera to *P. pastoris* PAS8. Antibody dilutions were 1/2,000, 1/1,000, 1/500, 1/1,000, respectively.

tions 13–25. The fraction most enriched for peroxisome markers (fraction 16), had a sucrose density of 1.19 g/cm³. This agrees with published figures for the density of yeast peroxisomes purified on sucrose gradients (Hohfeld et al., 1991).

Fig. 10 summarizes experiments performed on sucrose gradient fractions from a peroxisome purification gradient from Δ *pas1* cells. Similar to the wild-type gradient, SDH activity was found in fractions 28–37, with peak activity in fraction 31 (Fig. 10 a). Catalase activity (Fig. 10 a), how-

ever, was not found at all in fractions 13–25, and the only detectable catalase was a small amount found in fractions 37–40 (corresponding to free protein). An immunoblot with anti-thiolase antibody (Fig. 10 d) demonstrated that no thiolase was loaded on the gradient (even a very long ECL exposure did not show any thiolase on this blot; data not shown). These results suggest that the Δ *pas1* cells did not import thiolase and catalase into membrane-bound structures.

Blotting Δ *pas1* gradient fractions with antibodies that recognize the trifunctional protein (Fig. 10 b) and acyl-CoA

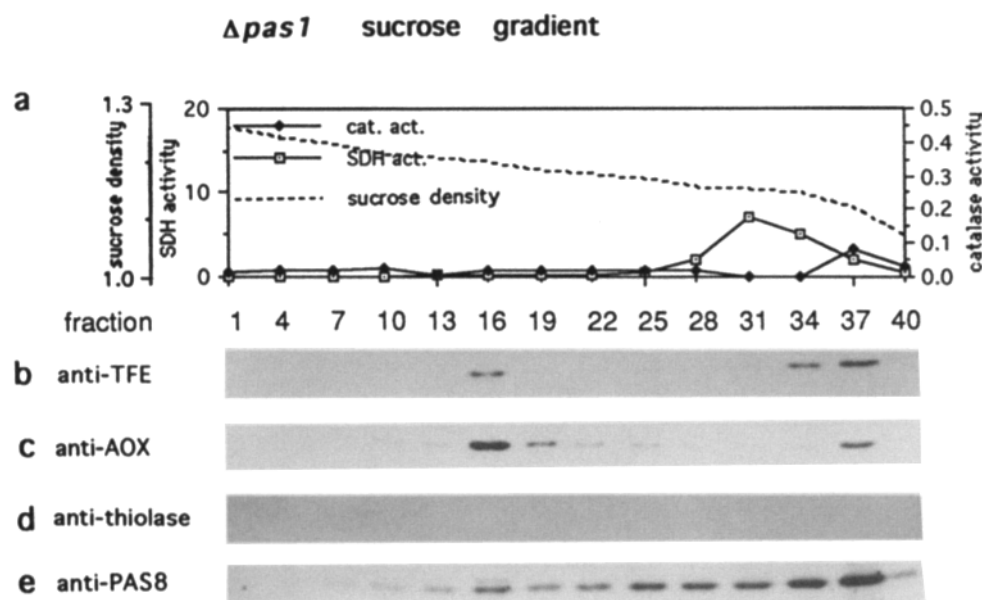


Figure 10. Purification of peroxisomes from Δ *pas1* cells on a sucrose gradient. Δ *pas1* cells were cultivated in oleic acid for 18 h and the organelle pellet fraction (8 mg) was fractionated on a sucrose gradient as described by Nuttley et al. (1990). (a) Gradient fractions assayed for catalase and SDH activities across the gradient. Density of fractions in g/cm³. Catalase, SDH, and Acyl-CoA oxidase activities are expressed as described in the legend to Fig. 9. (b–e), results of Western blotting equal volumes (35 μ l) of every fourth gradient fraction (including the first and last fractions) with: (b) antisera to trifunctional enzyme (anti-TFE); (c) antisera to acyl-CoA oxidase (anti-AOX); (d) antisera to thiolase; and (e) affinity-purified antisera to *P. pastoris* PAS8. Antibody dilutions were as described in the legend to Fig. 9.

oxidase protein (Fig. 10 c) revealed that both proteins concentrate in two distinct parts of the gradient: fractions 34–40, which are likely to contain free protein, and fractions 13–19, which correspond (both in number and in sucrose density) to the peak peroxisome-containing fractions from the wild-type gradient (Fig. 9). A blot with anti-PAS8 antibody (Fig. 10 e) shows that the PAS8 protein was distributed through the gradient in a bimodal manner, as was seen for the wild-type gradient. The comigration of acyl-CoA oxidase, PAS8 protein, and the trifunctional enzyme to fraction 16 is striking, especially in light of the fact that both the wild-type gradient fraction 16 (peak peroxisomal fraction) and $\Delta pasl$ fraction 16 have a density of 1.19 g/cm³.

The colocalization of these peroxisomal markers to fractions of 1.19 g/cm³ sucrose density in the gradient from $\Delta pasl$ cells was most likely due to one of three reasons: (a) the three proteins were part of a large protein complex that had sedimented to sucrose of 1.19 g/cm³ during the 8-h, 92,000-g spin; (b) the three proteins were loaded onto the sucrose gradient as a result of nonspecific clinging to the crude pellet, then during the spin migrated as free proteins to sucrose of density 1.19 g/cm³; or (c) the three proteins are associated with peroxisome ghosts.

To distinguish between the first and third possibilities, we performed a sucrose-flotation experiment (Walworth et al., 1989; Paravicini et al., 1992). Crude organellar pellets were prepared from oleic acid-induced wild-type and $\Delta pasl$ cells, then loaded on 5-ml sucrose gradients and spun for 2.5 h at 92,000 g. These gradients were fractionated and 200- μ l gradient material of the appropriate peroxisome- or peroxisome ghost-containing fraction was placed in a 5-ml high-speed centrifuge tube, overlaid with cushions (2.4 ml) of 60 and 35% sucrose and centrifuged at 170,000 g for 18 h in a swinging bucket rotor. Fractions were then assayed for the presence of peroxisomal markers. It was expected that protein aggregates that did not completely sediment during the first (8 h, 92,000 g) spin would completely sediment during the second (18 h, 170,000 g [Horazdovsky and Emr, 1993]) spin, and that membrane-enclosed proteins would rise to the 60/

35% sucrose interface (Stack et al., 1993). Since no mitochondria were loaded onto the flotation gradient (the material loaded onto this gradient contained no SDH activity; data not shown), there was no chance that proteins could rise to the 60/35% sucrose interface due to interaction with mitochondrial membranes.

In both wild-type and $\Delta pasl$ gradients, it is clear that the peroxisomal markers (trifunctional enzyme, acyl-CoA oxidase, and PAS8 protein) floated out of the densest sucrose and concentrated at the interface of the 60 and 35% sucrose (Figs. 11 and 12).

The fact that the peroxisomal markers behave similarly in the wild-type and the $\Delta pasl$ flotation gradients suggests that the colocalization of peroxisome markers in the $\Delta pasl$ purification gradient was not the result of the proteins being in a complex that had sedimented to sucrose of 1.19 g/cm³ during the 8-h, 92,000-g spin.

To test the possibility that acyl-CoA oxidase, trifunctional enzyme, and PAS8 migrated as free proteins to sucrose of density 1.19 g/cm³ in the $\Delta pasl$ purification gradient, we ran sucrose purification gradients with organellar pellets prepared from *P. pastoris* wild-type, $\Delta pasl$, and $\Delta pas8$ cells. The $\Delta pas8$ strain was used because it is known that it will not import acyl-CoA oxidase or trifunctional enzyme into peroxisomes: *P. pastoris* $\Delta pas8$, and the equivalent *S. cerevisiae* strain, *pas10*, are characterized by peroxisomes that fail to import PTS1-containing proteins and the PAS8 protein of *P. pastoris* is the PTS1 receptor (McCollum et al., 1993; van der Leij et al., 1993).

In the wild-type and the $\Delta pasl$ gradients, trifunctional enzyme and acyl-CoA oxidase were distributed essentially as in the gradients described in Figs. 9 and 10 (peroxisomal markers were found in peak levels in both gradients at density 1.21 g/cm³; data not shown) whereas in the $\Delta pas8$ gradient, no acyl-CoA oxidase was detected at all, and the very small amount of trifunctional enzyme detected was found at a density of 1.16 g/cm³ at the top of the gradient. Thus, in $\Delta pas8$ cells, acyl-CoA oxidase was not imported into peroxisomes, nor did it cling to the pre-gradient pellet in an amount

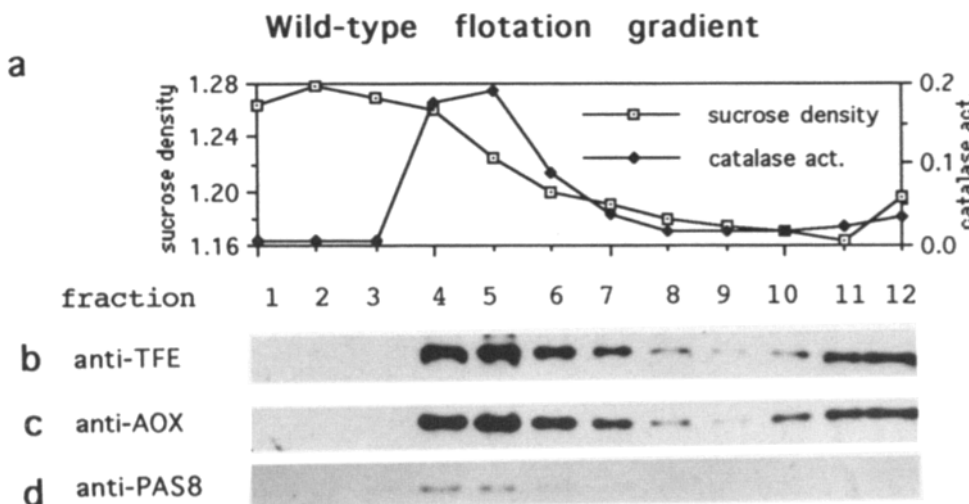


Figure 11. Localization of peroxisomal markers in two-step sucrose gradients. Wild-type cells were induced on oleic acid for 18 h and an organellar pellet fraction, consisting primarily of peroxisomes and mitochondria, was obtained. This pellet was loaded on a 5-ml sucrose gradient, spun for 2.5 h at 92,000 g, and fractionated into 12 fractions of ~ 480 μ l each. 200 μ l from a fraction of density 1.21 g/cm³ was transferred to the bottom of a Beckman ultra-clear centrifuge tube, overlaid with 2.4 ml 60% sucrose, then overlaid again with 2.4 ml 35% sucrose. This sample was centrifuged

at 170,000 g for 18 h and dripped from bottom (fraction 1) to top (fraction 12) in ~ 0.5 ml fractions. (a) Gradient fractions assayed for SDH activity across the gradient. Density of fractions in g/cm³. (b–d) Equal volumes (35 μ l) of fractions Western-blotted with the indicated antisera. Antibody dilutions are as described in the legend to Fig. 9.

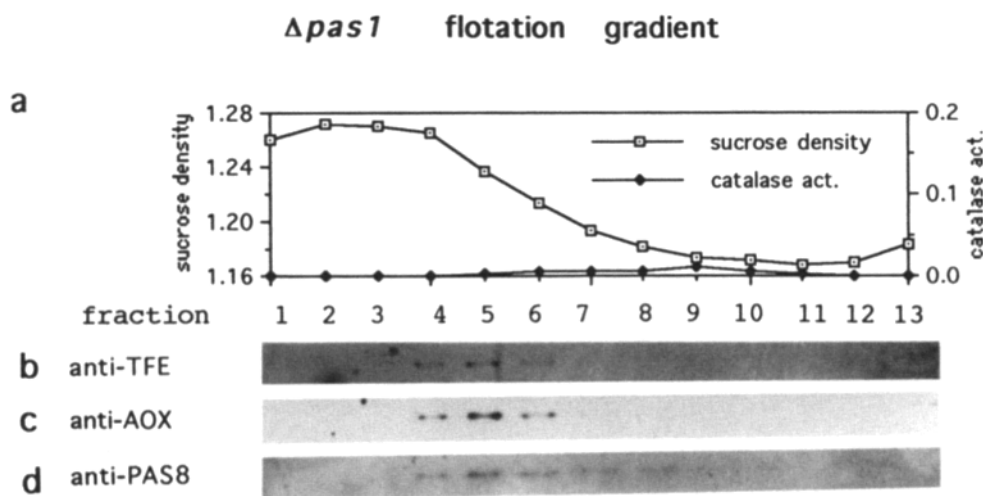


Figure 12. Localization of peroxisomal markers in two-step sucrose gradients. *Δpas1* cells were induced on oleic acid for 18 h and the organellar pellet fraction was subjected to the same treatments as the pellet fraction in Fig. 11. Fractions from the two-step sucrose gradient were dripped from bottom (fraction 1) to top (fraction 13) in ~0.5 ml fractions. (a) Gradient fractions assayed for SDH activity across the gradient. Density of fractions in g/cm³. (b–d) Equal volumes (35 μl) of fractions Western-blotted with the indicated antisera. Antibody dilutions are as described in the legend to Fig. 9.

great enough to allow its detection in the *Δpas8* sucrose gradient. The small amount of trifunctional enzyme that did get loaded on the *Δpas8* gradient did not migrate to the density of wild-type peroxisomes. These results rule out the possibility that the distribution of trifunctional enzyme and acyl-CoA oxidase seen in the *Δpas1* sucrose gradient described in Fig. 10 was due to the two proteins clinging non-specifically to the crude pellet and then migrating as free proteins to sucrose of density 1.19 g/cm³. This fact, coupled with the flotation gradient results, suggests that trifunctional enzyme and acyl-CoA are imported to membrane-bound structures in *Δpas1* cells. Furthermore, these membrane-bound structures are peroxisomes because they have the correct density and they contain several peroxisomal markers, but no SDH activity.

Δpas1 Peroxisomal Ghosts Contain Some, but Not All, of the Proteins Seen in Wild-type Peroxisomes

When Figs. 9 and 10 are compared, it can be seen that *Δpas1* peroxisomal ghosts were completely deficient for the import of thiolase and catalase. When care is taken (see Materials and Methods) to ensure that the band intensity of a particular marker protein in gradient fractions can be used to compare the relative levels of that protein in the *Δpas1* and wild-type gradients, it is clear that several proteins (trifunctional enzyme, PAS8 protein, and acyl-CoA oxidase) were indeed imported in the *Δpas1* cells, but at very low levels when compared to the levels seen in wild-type peroxisomes. This is well illustrated by the difference in band intensity seen for trifunctional enzyme in Western blots of the wild-type (Fig. 9 b) and *Δpas1* (Fig. 10 b) sucrose gradients (ECL-development time required to detect trifunctional enzyme on the *Δpas1* blot is sufficient to overexpose the band corresponding to this protein in the wild-type blot).

Fractions from wild-type and *Δpas1* gradients were also blotted with anti-SKL antibody. Since COOH-terminal -SKL tripeptides are used as peroxisomal targeting signals in yeast (Distel et al., 1992), it was expected that antibodies directed against these sequences would recognize proteins in purified wild-type peroxisomes. This was the case: four proteins

much enriched in the peroxisomal fractions were recognized by anti-SKL antibody (molecular masses: 97, 72, 38, and 31 kD). Interestingly, when fractions from a *Δpas1* sucrose gradient were blotted with anti-SKL antibody, only the 97-kD protein (now known to be trifunctional enzyme) and the 72-kD protein (now known to be acyl-CoA oxidase) were detected. The two other proteins (38 and 31 kD) recognized by the anti-SKL antibody were not detected in blots of the *Δpas1* gradient (data not shown). However, since these two proteins are seen at lower intensities than trifunctional enzyme or acyl-CoA oxidase in blots of wild-type peroxisomes, it is possible that small amounts of these proteins might have gone undetected in the peroxisomes from *Δpas1* cells.

Thus peroxisome ghosts from *Δpas1* cells are unable to import any of the proteins tested with the same efficiency as wild-type peroxisomes. Some peroxisomal proteins (thiolase, catalase and the 38- and 31-kD proteins) are undetectable in the organelles from *Δpas1* cells, but others (trifunctional enzyme, acyl CoA oxidase, and PAS8) are present at reduced levels, relative to that in wild-type cells.

It is interesting that the proteins found in association with *Δpas1* ghosts include both PTS1-targeted proteins (trifunctional enzyme, acyl-CoA oxidase) and at least one protein that is not targeted via this pathway (PAS8 has no PTS1 sequence [McCollum et al., 1993]). *Δpas1* cells appear to be deficient in the import of a PTS2-containing protein (thiolase), as well as some proteins that are either known to be imported via the PTS1 pathway (e.g., catalase, McCollum et al., 1993) or others that are recognized by the anti-SKL antibody and may therefore be imported via this pathway.

The PAS1 Protein Is Induced by Growth on Oleic Acid or Methanol

Crude *P. pastoris* lysates from glucose-grown cells were blotted with an antibody made against a GST-PAS1 fusion protein. A protein of the predicted size, 127 kD, was recognized (Fig. 13). This protein was seen in increased amounts in lysates from cells grown on oleic acid or methanol. This protein was not recognized in lysates from *pas1::ARG4* cells grown on glucose, oleic acid, or methanol, proving that the

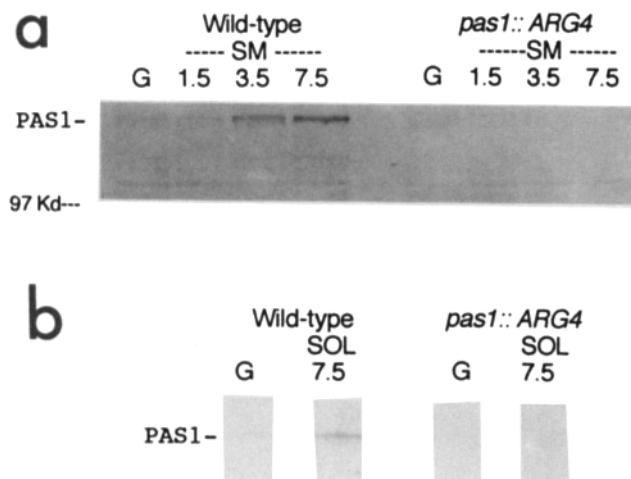


Figure 13. The PAS1 protein is induced by growth on oleic acid or methanol. Wild-type and *pas1::ARG4* cells were precultivated in SG medium and then shifted to SM medium for 1.5, 3.5, or 7.5 h, or to SOLT medium for 7.5 h. Glass-bead lysates were obtained from these cells. (a) 120 µg protein from methanol-induced cells was loaded in each lane. (b) 120 µg protein from oleic-acid induced cells was loaded in each lane. Western blotting was performed with antiserum raised against a fusion protein expressed by DNA encoding glutathione-S-transferase cloned upstream of an internal 2.4-kb BglII fragment of *PAS1*. Antisera was used at a 1/100 dilution.

recognized band was indeed the PAS1 protein. Thus, the *P. pastoris* PAS1 protein is induced by growth on oleic acid or methanol.

Discussion

The *P. pastoris* PAS1 Is a Homologue of the *S. cerevisiae* PAS1 and Is Required for Functional Peroxisomes

The evidence that the *P. pastoris* *PAS1* gene is required for peroxisomal biogenesis and is the homologue of the *S. cerevisiae* *PAS1* is as follows. (a) Disruption or deletion (by homologous recombination) of the *P. pastoris* *PAS1* results in cells that are unable to grow on oleic acid or methanol, carbon sources known to cause induction of wild-type *P. pastoris* peroxisomes and peroxisomal enzymes. In *S. cerevisiae*, oleic acid is the only carbon source known to induce peroxisomes and peroxisomal proteins (Kunau et al., 1988), and *S. cerevisiae* *pas1* cells are unable to grow on oleic acid as sole carbon source (Erdmann et al., 1989). (b) Electron micrographs of *P. pastoris* *pas1* cells induced on oleic acid or methanol show fewer and much smaller peroxisome-like structures than do similarly grown wild-type *P. pastoris* cells (Fig. 5, b and c). This is similar to results seen for *S. cerevisiae*, in which peroxisomal structures are never seen in oleic acid-induced *pas1* cells but are easily observed in induced wild-type cells. (c) Comparison of the predicted amino acid sequences reveals that the *P. pastoris* PAS1 shares 60% similarity and 39% identity with the *S. cerevisiae* PAS1 (Fig. 3). The sequences contain 1157 and 1043 residues, respectively, with homology throughout the length of the proteins and extremely high homology in two regions containing consensus ATP-binding domains.

Additionally, a search of GENBANK with the predicted *P. pastoris* PAS1 sequence reveals *S. cerevisiae* PAS1 as its best match in GENBANK. For comparison, *P. pastoris* PAS8 (McCollum et al., 1993) and its homologue, *S. cerevisiae* PAS10p, share 57% similarity and 37% identity (van der Leij et al., 1993). (d) Introduction of the cloned *P. pastoris* *PAS1* gene complements the peroxisomal defects seen in *P. pastoris* strains bearing a disruption or deletion of the chromosomal *PAS1* gene. (e) In Western blots, an antibody made against a GST-PAS1 fusion protein reacts with a protein of 127 kD. This protein is the correct size, it is induced on oleate and methanol (Fig. 13), it is absent in strains bearing a deleted or disrupted chromosomal *PAS1* gene, and it is over-expressed in strains bearing the *PAS1* gene on a multicopy episomal plasmid (relative to the amount of PAS1 seen in similarly grown wild-type cells; data not shown). (f) The *PAS1* genes of *P. pastoris* and *S. cerevisiae* are more closely related to each other (Fig. 3) than the *PAS1* and *PAS5* genes of *P. pastoris* (Fig. 4) which complement mutations that belong to different complementation groups, yet encode related proteins.

Ultimate proof that the *P. pastoris* *PAS1* and *S. cerevisiae* *PAS1* are functional homologues might be obtained if the gene from one organism complements a *pas1* mutant of the other. Unfortunately, we have been unable to show this. We have tried the following: (a) We cloned the *P. pastoris* *PAS1* gene, under its own promoter, into *S. cerevisiae* shuttle vector pRS316 and transformed this construct into an *S. cerevisiae* *pas1* strain. Cells bearing this construct did not regain the ability to use oleic acid. (b) We cloned the entire *S. cerevisiae* *PAS1* ORF behind the *P. pastoris* methanol oxidase promoter in a vector which contained a *P. pastoris* autonomous replication sequence and the *S. cerevisiae* *ARG4* gene. *P. pastoris* *pas1* cells harboring this construct did not grow on methanol. (c) We cloned, behind the *P. pastoris* *PAS1* promoter, a construct that encoded a fusion protein consisting of the first 83 amino acids of *P. pastoris* PAS1 fused to amino acids 123 to 1043 of *S. cerevisiae* PAS1 (residue 1043 is the final residue of *S. cerevisiae* PAS1). Cells containing this fusion construct were unable to grow on oleic acid or methanol. It should be noted, however, that homologous genes do not always function in a heterologous system.

Δpas1 Contains Peroxisomal Ghosts

Electron micrographs of oleic acid- or methanol-induced *Δpas1* cells contain structures that look like extremely small peroxisomes (Fig. 6, a and b) which are quite distinct morphologically compared to the peroxisomes seen under similar circumstances in wild-type cells (Fig. 5, b and c).

To confirm that *Δpas1* cells contain peroxisomal "ghosts," we purified these structures on sucrose gradients. Several peroxisome markers (acyl-CoA oxidase, trifunctional protein, and PAS8) comigrated to fractions of the same sucrose density (1.19 g/cm³) to which wild-type peroxisomes and peroxisomal markers migrate (Figs. 9 and 10). The colocalization, in *Δpas1* sucrose gradients, of bona fide peroxisomal markers at the same sucrose density as that of wild-type peroxisomes was not fortuitous. This was shown by the fact that both acyl-CoA oxidase and trifunctional enzyme, which in wild-type *P. pastoris* are imported into peroxisomes via the PTS1 pathway and migrate in sucrose organelle purification gradients to a density of 1.19 g/cm³, failed to localize

to this density in sucrose gradients loaded with an organellar pellet isolated from PTS1-import deficient $\Delta pas8$ cells (data not shown). The colocalization of multiple peroxisomal markers at a sucrose density of 1.19 g/cm³ in $\Delta pas1$ cells cannot be due to simple aggregation of these proteins either, as demonstrated by the fact that the markers floated to the interface between 60 and 35% sucrose during the $\Delta pas1$ sucrose flotation gradient experiment (Fig. 12) in a fashion virtually identical to that seen for markers enclosed in wild-type peroxisomes (Fig. 11). These observations provide strong evidence for the association and import of acyl CoA oxidase and trifunctional enzyme into peroxisome ghosts of $\Delta pas1$ cells. The presence of PAS8, the putative PTS1 receptor, in peroxisome ghosts of $\Delta pas1$ cells is consistent with their ability to import small quantities of certain PTS1-containing proteins.

The existence of detectable peroxisome ghosts in the $\Delta pas1$ mutant of *P. pastoris* clearly distinguishes it from the analogous mutant of *S. cerevisiae* (Erdmann et al., 1991). It is possible that these ghosts are more visible by electron microscopic analysis and more stable during biochemical manipulations in *P. pastoris*, relative to *S. cerevisiae*. The existence of these ghosts however, provides a satisfying explanation for the fact that peroxisomes reappear in the *pas1* mutants complemented with the *PAS1* gene from the corresponding organism (Fig. 6, c and d). If peroxisomes arise only from preexisting peroxisomes, and if the *pas1* mutant of *S. cerevisiae* really has no peroxisome ghosts, then it would be difficult to explain how peroxisomes are restored by the introduction of the *PAS1* gene into the *pas1* mutant of *S. cerevisiae*.

***Δpas1* Peroxisomal Ghosts Import Some, but Not All, of the Proteins Seen in Wild-type Peroxisomes**

The phenotype of the *S. cerevisiae pas1* mutant, i.e., cells with no detectable peroxisomes, has been explained as the result of a defect in protein import into peroxisomes (Erdmann et al., 1991). A more detailed analysis of the *pas1* mutant of *P. pastoris* shows that the situation is more complex. A comparison of data obtained from Western blots of wild-type and $\Delta pas1$ peroxisome purification gradient fractions confirmed that $\Delta pas1$ cells contain ghosts, and that these ghosts did not contain wild-levels of peroxisomal proteins (Figs. 9 and 10). Interestingly, however, a peroxisomal protein's import-fate in $\Delta pas1$ cells does not seem to be dependent on the protein's targeting signal alone: proteins absent from $\Delta pas1$ peroxisomes include the PTS1-targeted catalase and the PTS2-targeted thiolase, while proteins that are imported into $\Delta pas1$ peroxisomes include two PTS1-targeted proteins (trifunctional enzyme, which is recognized by anti-SKL antibody, and acyl-CoA oxidase, which is recognized by anti-AKI antibody and anti-SKL antibody) and PAS8, which does not contain a PTS1 or PTS2 (McCollum et al., 1993).

These results argue against PAS1 having a direct role in the import of proteins bearing a specific type of peroxisomal targeting signal: if this were the case, it would be expected that $\Delta pas1$ peroxisomes would contain wild-type amounts of all proteins except those targeted by a PAS1-requiring pathway. We have not yet determined the PAS1 protein's cellular location, but cellular fractionation suggests that PAS1 is not a free cytosolic protein. Additionally, Western blots of

sucrose-gradient purified peroxisomes isolated from oleic acid-induced, wild-type *P. pastoris* revealed no PAS1 protein associated with peroxisomes.

Role of the PAS1 Protein in Peroxisome Biogenesis

P. pastoris pas1 cells contain extremely small peroxisomes that will not support growth on oleic acid or methanol. The fact that $\Delta pas1$ cells contain peroxisomal ghosts suggests that *P. pastoris* PAS1 is not required for distribution of peroxisomes to daughter cells. Additionally, *pas1::ARG4* cells (which are phenotypically equivalent to $\Delta pas1$ cells) produce peroxisomal proteins at wild-type levels (Fig. 8), suggesting that PAS1 is not involved in the induction of peroxisomal proteins. Further, it is unlikely that a defect in peroxisomal substrate import would result in extremely small peroxisomes. Thus, the $\Delta pas1$ phenotype is likely to be due to a defect in peroxisomal matrix protein import or in the addition of membrane to growing peroxisomes.

The following characteristics of the $\Delta pas1$ ghosts lead us to believe that PAS1 functions in the addition of membrane to peroxisomes: (a) electron micrographs reveal that induced $\Delta pas1$ cells often contain structures that look like small peroxisomes. Similar structures are rarely seen in uninduced or fully induced wild-type cells. Interestingly, structures similar to the small, stacked organelles seen in the methanol-cultured $\Delta pas1$ cells (Fig. 6 a) are regularly seen in methanol-cultivated $\Delta pas5$ cells and in wild-type cells cultivated for short periods (less than 2 h) in methanol. This suggests that the peroxisome ghosts seen in the $\Delta pas1$ cells may be normal intermediates in the peroxisome biogenesis pathway and the situation is reminiscent of the immature acyl CoA oxidase-containing peroxisomes of rat liver cells, which import other proteins to become mature peroxisomes (Heinemann and Just, 1992; Luers et al., 1993). (b) Peroxisomal ghosts isolated from $\Delta pas1$ cells contain several, but not all, of the proteins seen associated with wild-type peroxisomes. Since peroxisomal proteins of at least two types (matrix proteins targeted via the PTS1 pathway and at least one membrane protein [PAS8] targeted via a different signal) are associated with purified ghosts, it does not seem likely that *pas1* cells are simply defective for matrix protein import. It should be noted that $\Delta pas1$ cells have fewer and smaller peroxisomes than wild-type cells under analogous peroxisome-induction conditions. The reduced efficiency of import of markers such as acyl-CoA oxidase, trifunctional enzyme or PAS8p, into peroxisomes of $\Delta pas1$ cells could be due, at least in part, to the possibility that these organelles can only accommodate limited amounts of matrix proteins. If they become full, and if the organelle cannot grow in size due to some other limitation, then there would appear to be a protein import defect.

These results are consistent with PAS1 playing a role in membrane addition: if peroxisomes could import protein but not accrue membrane material such as lipid or protein, cells growing on oleic acid or methanol would soon fill their peroxisomes to capacity with protein. Also, if it is posited that different peroxisomal proteins are imported to or retained in peroxisomes at different efficiencies, then peroxisomal proteins would be expected to collect in peroxisomal ghosts at differing levels.

A membrane-addition role for the PAS1 protein is consistent with the results found for *S. cerevisiae pas1*. This mutant

had no detectable peroxisomes, but Erdmann et al. (1991) mentioned that diminished peroxisomes might have been present and simply not detected. Additionally, they stated that the *S. cerevisiae pas1* defect could be complemented by the addition of the *PAS1* gene and that, if *S. cerevisiae pas1* cells contained no peroxisomes, this result would be inconsistent with the dogma that peroxisomes must come from preexisting peroxisomes.

It was mentioned earlier that *P. pastoris* PAS1 and PAS5 (Spong and Subramani, 1993) share considerable homology. Thus, it should be possible to explain the *pas5* phenotype as the result of a defect in membrane addition. This is the case because the *pas5* phenotype is similar to the *pas1* phenotype: cells from each strain contain small (with respect to wild type) peroxisomes and these peroxisomes can import some peroxisomal proteins (Spong and Subramani, 1993) but not others. Thus, the results reported for the *pas5* mutant are not inconsistent with a peroxisomal membrane addition role for PAS5.

Finally, the fact that SEC18 (Eakle et al., 1988) and its mammalian homologue NSF (Wilson et al., 1989) are thought to play a role in the intracellular trafficking of membrane-bound vesicles suggests that PAS1 may also be involved in intracellular membrane movement.

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